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 DE FR GB IT(71) Applicant: Matsushita Electric Industrial Co.,
 Ltd.
 1006, Oaza Kadoma
 Kadoma-shi Osaka-fu, 571(JP)(72) Inventor: NANKAI, Shiro
 50-12, Nasuzukuri 4-chome
 Hirakata-shi Osaka 573(JP)
 Inventor: KAWAGURI, Mariko
 Thowamoriguchihiataun 202 12-1,
 Dainichicho
 1-chome Moriguchi-shi Osaka 570(JP)
 Inventor: FUJITA, Mayumi
 131-85, Kajimachi 3-chome
 Moriguchi-shi Osaka 570(JP)
 Inventor: IIJIMA, Takashi
 35-26, Yamanouenishimachi
 Hirakata-shi Osaka 573(JP)(74) Representative: Graf von Wengersky, Anton et
 al
 Patentanwälte Leinweber & Zimmermann
 Rosental 7
 D-8000 München 2(DE)

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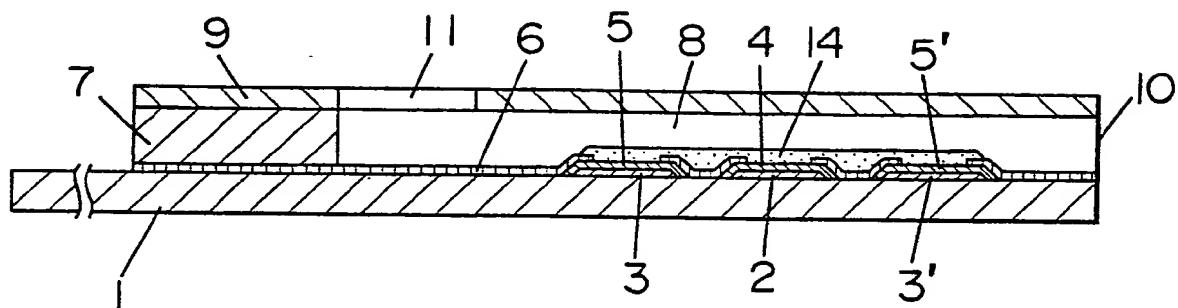
EP 0 359 831 A1 (54) BIOSENSOR AND PROCESS FOR ITS PRODUCTION.

(57) A biosensor of the invention comprises an insulating base board (1) having formed thereon, in sequence, leads (2, 3, 3'), an electrode system mainly made of carbon (4, 5, 5'), an insulating layer (6) and a reaction layer (14) composed of an enzyme and an electron acceptor, and being provided thereon with a space (8) defined by a spacer (7) and a cover (9). When a biological sample solution is

brought into contact with the inlet (10) of the biosensor having the above-described structure, the sample solution is introduced into its inside, while the air within the space (8) is rapidly discharged through the outlet (11) and, at the same time, the space (8) is filled with the sample solution up to the neighborhood of the outlet. Thus, measurement can be conducted inexpensively at a high speed with a high

accuracy through simple procedures without residual bubbles.

FIG. 6



SPECIFICATION

1 TITLE OF THE INVENTION

BIOSENSOR AND A PROCESS FOR PREPARATION
THEREOF

FIELD OF THE INVENTION

5 The present invention relates to biosensors which can quantitatively determine a specific component in various sample solutions from the living body in a rapid and easy way with high accuracy and a process for preparation thereof.

10 BACKGROUND OF THE INVENTION

In recent years, various biosensors utilizing a specific catalytic action possessed by enzyme have been developed and in particular, it has been attempted to apply biosensors to the clinical field. In the 15 present days when inspection items and sample numbers are increasing, biosensors which can provide rapid assay with good accuracy have been desired.

Taking a glucose sensor as an example, diabetes has markedly increased nowadays and for 20 measurement and control of blood sugar level in blood, it takes a very long time, since blood is centrifuged and plasma is provided for the measurement as is conventionally done. Thus, a sensor which can make

1 measurement with whole blood is required. As a handy
type, there is a stick-like support having provided
thereon a carrier containing an enzyme capable of
reacting only with glucose and a dye which causes a
5 change upon enzyme reaction or by the product of the
enzyme reaction, like a test sheet used for inspection
of urine. The stick takes the system that blood is
dropped onto the carrier and after a definite period of
time, a change of the dye is visually or optically
10 determined. However, interference is serious because of
colored matters in blood, resulting in poor accuracy.

Now, a multilayer type analysis carrier as
shown in Fig. 1 is proposed (Japanese Utility Model
Application Laid-Open No. 54-178495). The carrier has
15 the construction comprising a transparent support 51
having provided thereon, in order, a reagent layer 52, a
spreading layer 53, a waterproofing layer 54 and a
filtering layer 55. The measurement takes the following
system: when a blood sample is dropped from the upside,
20 solid components in blood such as red blood cells,
platelets, etc. are removed by the filtering layer 55,
the blood uniformly permeates into the spreading layer
53 through a hole 56 in the waterproofing layer and a
reaction proceeds in the reagent layer 52. After
25 completion of the reaction, a light is irradiated from
the arrow direction through the transparent support 51,
whereby a substrate concentration is determined by
colorimetry. The system takes a complicated

1 construction as compared to the conventional handy
stick-like carrier but its accuracy has improved because
blood cells are removed, etc. However, it takes a long
time for the permeation of blood and the reaction so
5 that the waterproofing layer 54 that prevents drying of
the sample is required. In addition, incubation at a
high temperature is required for accelerating the
reaction. Thus, the system involves problems that
apparatuses and carriers become complicated.

10 On the other hand, as the system for quantitative assay of a specific component in a sample such as blood, etc. from the living body with high accuracy without performing operations such as dilution, agitation, etc. of the sample solution, a biosensor as shown
15 in Fig. 2 has been proposed (for example, Japanese Patent Application Laid-Open No. 59-166852). The biosensor comprises an insulating base plate 63 having embedded therein an electrode for measurement 64 and a counter electrode 65 made of platinum, etc., having leads 61 and 62, respectively, and the exposed areas of these electrodes are covered with a porous material 66 having carried thereon an oxidoreductase and an electron acceptor. When a sample solution is dropped onto the porous material, the oxidoreductase and the electron
20 acceptor are dissolved in the sample solution, whereby an enzyme reaction proceeds with a substrate in the sample solution and the electron acceptor is reduced.
25 After completion of the reaction, the reduced electron

1 receptor is electrochemically oxidized and a substrate
concentration in the sample is determined from a current
level for the oxidation obtained in this case. In such a
construction, however, the electrodes require operations
5 such as washing, etc., while the porous material can be
exchanged for every assay thereby to readily provide for
measurement. On the other hand, if it is possible to
dispose the apparatus including the electrode system for
every measurement, operations for the measurement become
10 extremely simple but from aspects of electrode materials
such as platinum, etc., construction and the like, the
apparatus is very expensive unavoidably. For the
construction of platinum electrodes, the sputtering
method or the deposition method can also be used but
15 production costs increase.

As a disposal system including the electrode
system, a biosensor described in Japanese Patent
Application Laid-Open No. 61-294351 has been proposed.

As shown in Fig. 3, in this biosensor, the electrode
20 systems 72 (72'), 73 (73') and 74 (74') composed of
carbon, etc. are formed on an insulating base plate 71
by means of screen printing, etc.; after providing an
insulating layer 75 thereon, the electrode systems are
covered with a porous material 77 having carried thereon
25 an oxidoreductase and an electron acceptor and the whole
is integrated with a holding frame 76 and a cover 78.
When a sample solution is dropped onto the porous
material, the oxidoreductase and the electron acceptor

- 1 are dissolved in the sample solution, whereby an enzyme
reaction proceeds with a substrate in the sample
solution and the electron acceptor is reduced. After
completion of the reaction, the reduced electron
5 acceptor is electrochemically oxidized and a substrate
concentration in the sample is determined from a current
level for the oxidation obtained in this case.

In the foregoing conventional construction,
the base surface including the electrode system is not
10 always uniformly wetted so that air bubbles remain
between the porous material and the base plate, whereby
a response current is affected or its reaction rate is
reduced in some occasion. Further when an ambient
humidity is low, moisture in a sample solution
15 evaporates off during measurement so that a change in
response is observed. Furthermore, when a substance
that is readily adsorbed to electrodes or a substance
such as ascorbic acid or the like that is easily
oxidized is present in a sample solution, response of
20 the sensor is affected by such a substance.

Therefore, as sensors for measuring a specific
component in a vital sample solution such as blood or
the like in a simple and rapid way with high accuracy,
sensors which can provide measurement data merely by
25 adding a trace amount of sample solution thereto without
diluting or quantitatively determining the sample
solution previously are desired. Furthermore,

1 disposable type sensors which are free of operations
such as washing, wiping, etc. are desired.

DISCLOSURE OF THE INVENTION

The biosensor of the present invention
5 comprises an insulating base plate having provided
thereon the electrode system comprised of at least an
electrode for measurement and a counter electrode and on
the electrode system a reaction layer containing an
enzyme and an electron acceptor. Further by integrating
10 with a cover, a space including the reaction layer is
formed and an introducing port for introducing a sample
solution into the space and a discharge port for
discharging a gas in the space by inflow of the sample
solution. A change of a substance in concentration
15 caused by the reaction between the enzyme and the sample
solution is detected by the electrode system thereby to
determine a substrate concentration in the sample
solution.

Furthermore, the electrode system may also be
20 comprised of a plural set of electrode systems and
reaction layers corresponding to the plural set of
electrode systems are provided, whereby the independent
sensor systems can be constructed. By doing so, two or
more substrate concentrations can be concurrently
25 determined. Furthermore, two pairs of electrode systems
may also be used, wherein one electrode system is used
for measurement and another electrode system is used for

1 blank. By determining a difference in response between
the electrode systems, interference by ascorbic acid,
etc. can be eliminated.

Further by integrating the base plate, the
5 electrode system and the reaction layer with the cove, a
space is formed and the surface of members constructing
the space is rendered hydrophilic, whereby a definite
quantity of sample solution can be readily introduced
into the space described above through a simple
10 operation merely by bringing a trace amount of sample
solution into contact with the introducing port of the
sensor.

The introduced sample solution dissolves the
electron acceptor, enzyme and hydrophilic high molecular
15 substance in the reaction layer, where an enzyme
reaction proceeds while converting the sample solution
into a viscous liquid. By the enzyme reaction, a
reduced electron acceptor is produced in response to the
substrate concentration in the sample solution. Then,
20 the reduced electron acceptor is electrochemically
oxidized on the electrode for measurement to determine
the substrate concentration from a current level for the
oxidation.

In the biosensor of the present invention, a
25 hydrophilic high molecular substance is used so that
influence of solid components such as red blood cells
and the like in a blood sample on the electrodes can be
prevented and at the same time, by providing the space,

1 fluidization of the sample solution on the electrodes
can be effectively prevented. Thus, an influence by
vibration during measurement can be prevented.

On the other hand, the reaction layer in the
5 biosensor of the present invention is comprised of an
enzyme layer and an electron acceptor layer provided
thereon. Further by providing a hydrophilic high
molecular substance layer between these layers, the
enzyme and the electron acceptor are separated from each
10 other to provide a biosensor having excellent
preservation property.

According to the present invention, a
disposable biosensor including the electrode system can
be constructed so that a substrate concentration in the
15 sample, for example, a glucose concentration in blood
can be determined rapidly in a simple operation with
high accuracy, merely by adding an extremely trace
amount of sample solution, without diluting or
quantitatively determining the sample solution in
20 advance.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an illustrative drawing showing an
example of conventional glucose sensors. Figs. 2 and 3
illustratively show glucose sensors using conventional
25 enzyme electrodes. Fig. 4 shows a perspective view of a
disassembled biosensor which is one embodiment of the
present invention. Fig. 5 shows an external view of the

1 biosensor. Fig. 6 illustratively shows a cross-
sectional view of the biosensor. Fig. 7 shows a
response characteristic of the biosensor. Fig. 8 shows
a perspective view of a disassembled biosensor which is
5 another embodiment of the present invention. Fig. 9
shows an external view of the biosensor. Figs. 10, 12
and 13 show perspective views of disassembled biosensors
which are other embodiments of the present invention.
Fig. 11 shows a perspective view of a disassembled
10 biosensor which is another embodiment of the prior art.
Fig. 14 shows a relationship between ascorbic acid
concentration and response characteristic. Fig. 15
shows a relationship between the particle diameter of
potassium ferricyanide and the time when the reaction is
15 completed.

BEST MODES FOR PRACTICING THE INVENTION

Example 1

In the following explanatory drawings in the
examples, the same numbering is used for common elements
20 and their explanation is in part omitted.

As one embodiment of the biosensor, a glucose
sensor is explained. Fig. 4 shows a perspective view of
a disassembled glucose sensor prepared as one embodiment
of the biosensor in accordance with the present
25 invention. Fig. 5 shows an external view of the
biosensor. Fig. 6 illustratively shows a cross-
sectional view of the biosensor obtained when the

1 biosensor shown in Fig. 5 is cut at the center into the
length direction. In Fig. 4, a reaction layer 14 shown
in Fig. 6 is not shown.

Hereafter a process for preparing the sensor
5 is described. Silver paste is printed on an insulating
base plate 1 composed of polyethylene terephthalate by
means of screen printing to form leads 2, 3 (3'). Next,
conductive carbon paste containing a resin binder is
printed thereon. By drying with heating, the electrode
10 system comprised of an electrode for measurement 4 and a
counter electrode 5 (5') is formed. Furthermore,
insulating paste is printed so as to partly cover the
electrode system to make the exposed area of the
electrodes definite and cover unnecessary part of the
15 leads. By a heat treatment, an insulating layer 6 is
formed.

Next, the exposed area of the electrode system
4, 5 (5') is polished and then heat-treated at 100°C for
4 hours in the air. After the electrode portion is thus
20 constructed, 0.5% aqueous solution of carboxymethyl
cellulose (hereafter simply referred to as CMC) as the
hydrophilic high molecular substance is spread onto the
electrodes and dried to form a CMC layer. A solution of
glucose oxidase (GOD) as the enzyme in phosphate buffer
25 solution is spread thereon and dried to form a reaction
layer 14 comprised of CMC-GOD layer. In this case, CMC
and GOD form a thin layer having a thickness of several
microns in a partly mixed state.

1 Next, three members of this base plate 1, a
spacer 7 comprised of a resin plate and a cover 9 are
adhered to each other in such a positional relationship
between the respective members shown by broken lines as
5 shown in Fig. 4 to integrate the sensor as shown in the
external view in Fig. 5. Herein, the spacer has a
thickness of approximately 300 μm and takes a shape that
the central part is cut off into a U-(dai) shape having
a width of 2 (dai) and a length of 7 mm. The end of the
10 cut portion becomes an introducing port 10 of a sample
solution and the central part forms a space 8, when
integrated. Furthermore, the cover 9 possess a hole
having a diameter of 2 (dai) and forms a discharge port
11 when integrated.

15 When the introducing port at the tip of the
glucose sensor constructed as described above is brought
into contact with a glucose standard solution (200
 mg/dl) which is a sample solution, the sample solution
is introduced into the inside through the introducing
20 port 10. In this case, the air in the space 8 is
rapidly discharged through the discharge port 11 and at
the same time, the space is filled up with the sample
solution up to near the discharge port. As such, the
sample solution rapidly spreads onto the electrode
25 surface to fill up the space so that any remaining air
bubbles are not noted.

This is believed to be because the sample
solution would flow into one direction by providing the

1 introducing port and the discharge port and due to the hydrophilic high molecular substance layer previously formed on the electrodes, wetting on the electrode surface would be improved so that the gas is smoothly
5 exchanged with the liquid.

Further by previously treating the surfaces of the members constructing the space such as the cover, spacer, etc., with a surface active agent to render them hydrophilic, the sample solution can be introduced more
10 smoothly.

On the other hand, the added sample solution dissolves CMC therein to render the liquid viscous. Glucose in the sample solution reacts with the enzyme by the action of glucose oxidase carried on the electrodes
15 to produce hydrogen peroxide. Therefore, by applying a voltage of 1 V between the electrodes one minute after introduction of the sample solution, the electrode for measurement is polarized into the anode direction. By applying a voltage to the anode direction described
20 above, an oxidizing current for the produced hydrogen peroxide is obtained. This current level corresponds to the concentration of glucose which is a substrate.

As an example of the response characteristic of the sensor constructed as described above, the
25 relationship between a current level 5 seconds after application of voltage and a glucose concentration is shown in Fig. 7, A, indicating that a good response characteristic was obtained.

1 Further with respect to 30 glucose sensors having the same specification as described above, the same sample solution was tested with the sensors. A coefficient of variation was as good as about 5%.

5 Furthermore, influence by evaporation was examined under humidity conditions of 30% and 80%, using 10 sensors each, and compared with the glucose sensor shown in Fig. 3 which was prepared in a conventional construction by way of trial. In the glucose sensors 10 according to the present invention, the influence was negligible even after allowing to stand for 5 minutes. However, in the sensors having a conventional construction, the evaporation was higher by about 3% under the lower humidity condition 2 minutes after.

15 As described above, the glucose sensor of the present invention having the construction described above can rapidly absorbs a trace amount of the sample solution into the reaction layer zone and can determine a glucose concentration in a simple and rapid way with 20 high accuracy, without being affected by ambient humidity, etc.

Example 2

The procedure was quite the same as in Example 1 until the CMC-GOD layer was formed. Thereafter, a 25 reaction layer composed of a CMC-GOD-electron acceptor layer was further formed thereon by the following method.

1 Surface active agent, lecithin (phosphatidyl
choline), was dissolved in toluene to prepare 1 wt%
solution and microcrystals (a mean particle diameter of
less than 10 μ mm) of potassium ferricyanide as an
5 electron acceptor was dispersed in the solution. Then
toluene was evaporated off to form the reaction layer
composed of CMC-GOD-electron acceptor layer on the
electrode system followed by integrating with a spacer
and a cover as in Example 1. Thus, a glucose sensor
10 having the construction shown in Fig. 5 was obtained.

Next, a glucose standard solution was introduced through the tip portion of the sensor as described above. By applying a voltage of 600 mv between the electrodes about one minute after, potassium ferricyanide produced by the enzyme reaction was oxidized on the electrode for measurement. A current level in this case was measured 5 seconds after application of the voltage. As the result, an extremely good linear relationship was obtained between the
15 glucose concentration and the response current level, as shown in Fig. 7, B. Further using 30 sensors, a variation coefficient was examined. The variation coefficient was 2.6% with respect to the glucose standard solution and 3% with respect to the whole blood
20 sample, which were extremely good. Further with respect to influence of evaporation, a test was carried out in a manner similar to Example 1 and similar effects were
25 obtained, as described above.

In the above example, when the concentration of lecithin was greater than 0.01 wt%, potassium ferricyanide was efficiently dispersed in toluene so that dropping became easy to form a thin potassium ferricyanide-lecithin layer. Where no lecithin is present, defects that the potassium ferricyanide layer was non-uniformly formed or the base plate was peeled off when bent were noted. However, by incorporating lecithin, the potassium ferricyanide layer which was uniform and peeled off only with difficulty could readily be formed. As the concentration of lecithin increased, the potassium ferricyanide layer was peeled off more difficultly but a dissolution rate of potassium ferricyanide also decreased. Therefore, a suitable concentration is believed to be 0.01 to 3 wt%.

Polyethylene glycol alkyl phenyl ether (trademark: Triton X) was used instead of lecithin. In order to disperse fine particles of potassium ferricyanide in toluene, more than 0.1% was necessary but a good potassium ferricyanide layer could be formed as in the case of using lecithin. As the surface active agent, there are oleic acid, polyoxyethylene glycerine fatty acid ester, cyclodextrin, etc., in addition to the example described above. Surface active agents are not particularly limited so long as they can disperse the electron acceptor in an organic solvent and do not affect the enzyme activity. As the organic solvent for mixing the electron acceptor therewith, solvents such as

1 toluene, petroleum ether, etc. may be used as long as
they have a minimized influence on GOD activity and the
printed electrodes.

With respect to the particle diameter of
5 microcrystalline potassium ferricyanide used above,
commercially available crystals of potassium
ferricyanide were ground into powders and crystals of a
definite particle diameter were collected by sieving to
form a potassium ferricyanide layer. With respect to
10 the same glucose sensors as described above prepared
from crystals of various particle diameters, their
responses were compared with each other. Fig. 15 shows
a mesh size of sieve on the abscissa and on the
ordinate, a time for completing the reaction to 400
15 mg/dl of glucose. Numerals with parentheses indicate a
size (μm) of the mesh hole. As shown in Fig. 15,
crystals having a smaller particle size were dissolved
more quickly and the time required for completing the
reaction was shorter. In the sensor prepared with
20 potassium ferricyanide (particle size of 100 μm or
less) passed through 145 mesh (Japanese Industrial
Standard), the reaction was completed within 2 minutes.
In addition, when the potassium ferricyanide layer
was prepared, crystals having a smaller particle
25 diameter could form a uniform layer and provided less
unevenness in response. Microcrystals of potassium
ferricyanide could be formed by grinding into powders
but recrystallization of an aqueous potassium

1 ferricyanide solution from ethanol could easily prepare
crystals having a particle diameter of not greater than
10 µm. When the potassium ferricyanide layer was formed
from such crystals, the layer became dense and the time
5 for completing the reaction could be shortened.

When potassium ferricyanide finely divided
into a particle diameter of less than 100 µm was mixed
with toluene and the mixture was dropped, toluene was
rapidly evaporated and the potassium ferricyanide layer
10 could be formed in a microcrystalline state so that a
dissolution rate was rapid and rapid measurement was
attained. Further by using the organic solvent, the
potassium ferricyanide layer could be formed separately
on the CMC-GOD layer, whereby preservation property
15 could be improved.

In providing the introducing port and the
discharge port in the example described above, in
addition to the arrangement shown in Figs. 4 through 6,
holes may be provided in the cover 9 and the base plate
20 1, respectively, and used as the introducing port 10 and
the discharge port 11 as shown in the perspective view
of the disassembled sensor in Fig. 8 and in the external
view in Fig. 9.

Furthermore, as shown in the perspective view
25 of the disassembled sensor in Fig. 10, the spacer may be
divided into two parts of 7 and 7' and the parts may be
used as the spacer 8, and the introducing port 10 and
the discharge port 11.

1 Furthermore, the shape of the tip portion of
the biosensor having the structure shown in Fig. 5 was
rounded as shown in the external view shown in Fig. 11.
By doing so, a portion brought into contact with a
5 sample solution was limited almost to the introducing
port 10 when the sample solution was introduced. Thus,
the sample solution did not go around the periphery of
the introducing port but could be smoothly introduced
even in a small quantity.

10 Example 3

The electrode portion shown in Fig. 12 was
constructed in a manner similar to Example 1. That is,
silver leads 3, 21, 22 and 23, counter electrode 3
corresponding thereto and three electrodes for
15 measurement 41, 42 and 43 were formed on a base plate to
construct three pairs of electrode systems possessing
the counter electrode in common. Next, a reaction layer
composed of CMC-GOD-electron acceptor layer was formed
around each of the electrodes for measurement in a
20 manner similar to Example 2.

Then, a spacer and a cover were adhered to
integrate them as in Example 1. Herein, the spacer is
partly cut off into a "comb-like" shape and the cut
portion forms a space 8. The space is divided into the
25 respective electrode systems but is continuous as a
whole to form a common space. Furthermore, the end
becomes the introducing port 10.

1 In response to the space dividedly constructed
on these respective electrode systems, three holes are
formed in the cover 9 to provide discharge ports 11, 12
and 13. When the introducing port 10 is brought into
5 contact with a sample solution, the sample solution is
rapidly introduced into the space, while discharging the
air in the space through the three discharge ports.

A response to the glucose standard solution
was measured with respect to the three electrode systems
10 in a manner similar to Example 2 and its mean value was
determined to make it a response level for one sensor.
The test was performed with 30 glucose sensors having
the same specification. The variation coefficient was
as good as 2%.

15 In the disposable type sensor, it is important
for improved reliability to minimize factors for error
including a difference in electrode area of each sensor.
The difference between the sensors forms a cause for
causing a difference in error of measurement data on the
20 same sample solution. However, as described above,
measurement with higher accuracy can be rapidly achieved
by providing a plurality of electrode systems for the
same sensor and obtaining a mean value of the response
levels.

25 Fig. 12 shows the embodiment having 3 pairs of
electrodes but the present invention is not limited
thereto and can further provide a large number of
electrode systems. Furthermore, the shape of the space

1 is not limited to those shown in the figures, likewise
the shape or arrangement of the electrode systems.

Example 4

Fig. 13 shows a variation of the construction
5 having a plurality of the electrode systems. In a
manner similar to Example 1 already described, silver
leads 21, 22 and 3 and two pairs of the electrode
systems comprised of electrodes for measurement 41 and
42 corresponding thereto and a counter electrode 5 in
10 common were constructed on the base plate. Next, as in
Example 2, after a reaction layer composed of CMC-GOD-
potassium ferricyanide layer was formed on each of the
electrode systems, a spacer 7 and a cover 9 were adhered
thereto. Herein, the spacer has two holes which
15 independently form spaces 81 and 82 and, discharge ports
11 and 12 corresponding to the holes and introducing
port 10 used in common are provided in the cover.

With respect to the glucose sensor having the
construction described above, as shown in Fig. 3, an
20 average was taken from response values of two pairs of
the electrode systems to the glucose standard solution
and made a measurement value for one sensor. When 30
sensors having the same specification were tested, the
variation coefficient was as good as 2.2%.

25 As such, in the sensor shown in Fig. 13,
measurement can be made with higher accuracy. In
addition, since the sensor possesses the independent

1 spaces, different sample solutions can be measured with
one sensor or the sensor can be provided for measurement
under different conditions in time, temperature, etc.

5 In the example described above, one hole was
provided in the cover to make it a common introducing
port but the present invention is not limited to this
embodiment. It is important to construct the
measurement system comprising a plural pairs of the
electrode systems and a plurality of independent spaces.
10 Therefore, two holes may also be provided and
introducing ports to the respective spaces may be
constructed.

Example 5

Using the electrode part, spacer and cover
15 having constructions shown in Fig. 13, a glucose sensor
described below was constructed. Firstly, a reaction
layer composed of CMC-GOD was formed on an electrode for
measurement 41 in a manner similar to Example 1. On the
other hand, CMC layer alone was formed on an electrode
20 for measurement 42.

With respect to the glucose sensor having two
pairs of the electrode systems obtained as described
above, a glucose standard solution (200 mg/dl)
containing ascorbic acid having various concentrations
25 was dropped onto introducing port 10 and introduced onto
each of the electrode systems. Next, as in Example 1, a
voltage of 1 V was applied about 1 minute after the

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
0 10 20 30 40 50 60 70 80 90

1 dropping and a current level was measured 5 seconds
after. The results are shown in Fig. 14. The output of
the electrode system of CMC-GOD layer is shown by A and
the output (blank output) of the electrode system of CMC
5 layer alone is shown by B. As is evident from the
drawing, the output of A increases as the concentration
of ascorbic acid increases and on the other hand, a
similar increase is noted with the output of B. This
indicates that the sensitivities of the respective
10 electrode systems to ascorbic acid are almost equal to
each other. When a difference in output between the
both electrode systems (A - B) is detected therefrom, a
current level based on glucose can be obtained. That
is, by using two pairs of the electrode systems, an
15 error due to substances sensitive to electrode can be
greatly reduced. Such an effect was also noted with
uric acid, etc., in addition to ascorbic acid.

As such, by constructing the sensor by
providing two pairs of the electrode systems and forming
20 a hydrophilic high molecular substance-enzyme layer on
one electrode system and a hydrophilic high molecular
substance layer alone on another electrode system, a
substrate concentration in the sample solution
containing interferants can be measured with good
25 accuracy.

In the above, after the CMC-GOD layer is
formed on both electrode systems, local heating by laser
or irradiation with ultraviolet rays, etc. may also be

1 applied only to either electrode system, whereby GOD is
inactivated to prepare the electrode system for blank
outputting. By doing so, the constructions are
identical in the two electrode systems except for enzyme
5 activity so that output currents due to interferants in
the two electrode systems can be conformable much better
with each other, resulting in an improved accuracy in
detection with the sensor.

In the foregoing embodiment, the electrode
10 system wherein the electrode portion comprises two
electrodes of the electrode for measurement and the
counter electrode has been described. By constructing
the electrode system by three electrodes further
involving silver/silver chloride, the accuracy can
15 further be improved. One embodiment for constructing
the electrode system comprises printing 3 silver leads
onto a base plate, then printing a carbon paste only on
the tip portions of two leads to coat an insulating
layer, treating the surface of the tip portion of the
20 remaining lead in which silver is exposed to form silver
chloride into a silver/silver chloride electrode. Thus,
the electrode system could be constructed in such a
manner.

Example 6

25 In Example 5, a reaction layer composed of
CMC-GOD-potassium ferricyanide layer and a layer
composed of CMC-potassium ferricyanide were formed on

1 the electrode for measurement 41 and the electrode for
measurement 42, respectively, in a manner similar to
Example 2. Measurement was performed in a manner
similar to Example 5 except that the voltage applied was
5 0.6 V. Influence of ascorbic acid could be removed as
described above.

Example 7

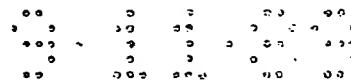
In Example 5, a reaction layer composed of CMC-GOD-potassium ferricyanide layer was formed on the
10 electrode for measurement 41 in a manner similar to Example 2. Further on the electrode for measurement 42, the CMC layer was formed and an enzyme layer composed of glucose oxidase, mutarotase and B-glucosidase was then formed thereon and an electron acceptor layer composed
15 of potassium ferricyanide was further formed to make a reaction layer. Measurement was performed by applying a voltage of 0.6 V and using as a sample solution an aqueous solution containing glucose and sucrose. With the electrode for measurement 41, a current in response
20 to glucose concentration was obtained and a current in response to the total concentration of glucose and sucrose was obtained with the electrode for measurement 42. From a difference between these current levels, the sucrose concentration could be determined. As such, two
25 substrate concentrations can be measured.

1 applied only to either electrode system, whereby GOD is
inactivated to prepare the electrode system for blank
outputting. By doing so, the constructions are
identical in the two electrode systems except for enzyme
5 activity so that output currents due to interferants in
the two electrode systems can be conformable much better
with each other, resulting in an improved accuracy in
detection with the sensor.

In the foregoing embodiment, the electrode
10 system wherein the electrode portion comprises two
electrodes of the electrode for measurement and the
counter electrode has been described. By constructing
the electrode system by three electrodes further
involving silver/silver chloride, the accuracy can
15 further be improved. One embodiment for constructing
the electrode system comprises printing 3 silver leads
onto a base plate, then printing a carbon paste only on
the tip portions of two leads to coat an insulating
layer, treating the surface of the tip portion of the
20 remaining lead in which silver is exposed to form silver
chloride into a silver/silver chloride electrode. Thus,
the electrode system could be constructed in such a
manner.

Example 6

25 In Example 5, a reaction layer composed of
CMC-GOD-potassium ferricyanide layer and a layer
composed of CMC-potassium ferricyanide were formed on



1 the electrode for measurement 41 and the electrode for
measurement 42, respectively, in a manner similar to
Example 2. Measurement was performed in a manner
similar to Example 5 except that the voltage applied was
5 0.6 V. Influence of ascorbic acid could be removed as
described above.

Example 7

In Example 5, a reaction layer composed of CMC-GOD-potassium ferricyanide layer was formed on the
10 electrode for measurement 41 in a manner similar to Example 2. Further on the electrode for measurement 42, the CMC layer was formed and an enzyme layer composed of glucose oxidase, mutarotase and B-glucosidase was then formed thereon and an electron acceptor layer composed of potassium ferricyanide was further formed to make a reaction layer. Measurement was performed by applying a voltage of 0.6 V and using as a sample solution an aqueous solution containing glucose and sucrose. With the electrode for measurement 41, a current in response
15 to glucose concentration was obtained and a current in response to the total concentration of glucose and sucrose was obtained with the electrode for measurement 42. From a difference between these current levels, the sucrose concentration could be determined. As such, two
20 substrate concentrations can be measured.

1 Example 8

After the electrode portion was prepared in a manner similar to Example 1, 0.5% aqueous solution of carboxymethyl cellulose (hereafter simply referred to as CMC) as the hydrophilic high molecular substance was spread onto the electrodes and dried to form a CMC layer. Next, a solution of glucose oxidase in water was spread thereon and dried to form a CMC-GOD layer. In this case, CMC and GOD formed a thin layer having a thickness of several microns in a partly mixed state. Furthermore, 0.5% ethanolic solution of polyvinylpyrrolidone (hereafter simply referred to as PVP) was spread so as to fully cover the first layer composed of this CMC-GOD layer and dried to form a second layer composed of PVP layer. A mixture of microcrystalline potassium ferricyanide as the electron acceptor and 1% solution of a surface active agent, lecithin, in toluene was dropped and spread onto the PVP layer and dried to form a third layer composed of potassium ferricyanide-lecithin layer followed by integrating with a spacer and a cover as in Example 1.

By using toluene in which PVP is sparingly soluble as a solvent in the case of forming the potassium ferricyanide-lecithin layer, it is possible to uniformly spread the potassium ferricyanide-lecithin solution onto the PVP layer. As the result, the uniform potassium ferricyanide-lecithin layer can be obtained.

As such, the use of a solvent in which the

- 1 hydrophilic high molecular substance constructing the second layer as the solvent for spreading the electron acceptor and the surface active agent could form the extremely uniform electron acceptor layer (third layer).
- 5 A glucose standard solution was introduced into the glucose sensor constructed as above through the introducing port. By applying a voltage of +0.6 V to the electrode for measurement in the anode direction one minute after, a response current was measured 5 seconds.
- 10 A good linear relationship was obtained up to the concentration as high as more than 900 mg/dl. Onto the glucose sensor described above, 5 μ l of blood sample was dropped and a response current was measured one minute after. A response with very good reproducibility was
- 15 obtained.

With respect to two sensors, i.e., the glucose sensor prepared by the process described above and the same glucose sensor except that the second layer composed of hydrophilic high molecular substance was not provided, preservation test was performed at 35°C for 30 days in a dried state. Using a glucose standard solution (90 mg/dl) as a sample solution, sensor response was compared 30 days after. The sensor in which the second layer composed of the hydrophilic high molecular substance was not formed showed CV value of 5.3 but the sensor in which the second layer composed of PVP showed CV value as extremely good as 2.5.

1 Example 9

The CMC-GOD layer and the PVP layer were prepared in a manner similar to Example 8. A mixture of microcrystalline potassium ferricyanide as the electron acceptor and 0.5% solution of a surface active agent, lecithin, in ethanol was dropped and spread onto the PVP layer and dried to form a potassium ferricyanide-lecithin layer. By using ethanol in which PVP is readily soluble as a solvent in the case of forming the potassium ferricyanide-lecithin layer, it is possible to concentratively develop at one point on the PVP layer. That is, it was possible to concentrate on the electrode for measurement of the sensor to construct the uniform potassium ferricyanide-lecithin layer so that the sensor in which a stable response was obtained merely by spreading the minimum quantity required could be prepared.

Response characteristics to the glucose standard solution of the glucose sensor constructed as above were determined in a manner similar to Example 8. A good linear relationship was obtained up to the concentration as high as more than 900 mg/dl. In addition, a response with very good reproducibility was obtained also in the case of using blood as a sample solution. Furthermore, with respect to the glucose sensor in which the second layer composed of hydrophilic high molecular substance was provided and the same glucose sensor except that the second layer composed of

1 hydrophilic high molecular substance was not provided,
preservation test was performed at 35°C for 30 days in a
dried state in a manner similar to Example 8. The
sensor in which the second layer composed of PVP showed
5 extremely good CV value, in the response measured 30
days after.

In the prior art construction, the GOD-CMC
layer was already in contact with the potassium
ferricyanide-lecithin layer at the time when the sensor
10 was prepared so that it was difficult to improve
preservation efficiency. The hydrophilic high molecular
substance layer composed of PVP which was used in
Example 8 described above and present Example 9 takes a
role to completely separate the GOD-CMC layer from the
15 potassium ferricyanide-lecithin layer in a dried state.

In addition, the hydrophilic high molecular
substance layer is extremely effective to ensure a
stable sensor response, also in the case that substances
readily adsorbed to the electrode or electrode-active
20 substances are present in a sample solution. Even in
the case that a glucose concentration was determined by
the glucose sensor described above using blood as a
sample solution, a stable sensor response was obtained
irrespective of viscosity or the like of the sample
25 solution.

In Examples 1 through 9 described above, one
embodiment of preferred positional relationship between
the introducing port and the discharge port connected

1 with the space is illustratively shown in the drawings. The discharge port may also be used as the introducing port and the introducing port may be used as the discharge port. Furthermore, the spacers and the covers
5 shown in the respective drawings are composed of independent materials but are not limited thereto. It is advantageous in mass production to form an integrated cover member with a shape of cover by means of molding or the like.

10 Further by rendering the surface of materials constructing the space hydrophilic, a sample solution was introduced more smoothly through the introducing port. The effect was obtained either by using hydrophilic materials or by previously treating the
15 surface materials constructing the space with a surface active agent to render them hydrophilic.

On the other hand, an interval between the base plate and the cover can be controlled by varying the thickness of spacer or the size of a portion
20 corresponding thereto. However, when the interval is too large, a quantity of sample solution required for filling up the space becomes large and it is also disadvantageous to introduce the sample solution by capillary phenomenon. When the interval is too small, a
25 resistance between the electrodes increases or a current distribution is distorted. From the foregoing, the interval is preferably in a range of 0.05 to 1.0

1 (dai), more preferably 0.1 to 0.5 (dai).

In the examples, CMC was used as the hydrophilic high molecular substance. As has already been stated, its role is to prevent influence of 5 adsorbable substances contained in a sample solution adsorbed to the electrode on response, in addition to the smooth introduction of the sample solution. Furthermore, its role also lies in separating the enzyme layer from the electron acceptor layer by the 10 hydrophilic high molecular substance layer thereby to further improve preservation property. As the hydrophilic high molecular substance, gelatin, methyl cellulose and the like can be used, in addition to CMC, and hydrophilic high molecular substances of starch, 15 carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride are preferred. These water-absorbing or water-soluble hydrophilic high molecular substances are dissolved in a suitable concentration and the resulting solution is 20 coated and dried to form a hydrophilic high molecular substance layer having a necessary layer thickness.

In the foregoing examples, the electrode system in which the electrode portion is comprised of two electrodes of an electrode for measurement and a 25 counter electrode has been stated. However, by constructing the electrode system with three electrodes further including a reference electrode, the accuracy can be further improved. As materials for the

1 electrode, carbon illustrated in the examples is suitable as a stable and inexpensive material but the materials are not limited thereto. Other noble metal materials and oxidized materials may also be used.

5 Further as the electron acceptor, though potassium ferricyanide used in the examples is excellent in stability and reaction rate, redox compounds such as quinone compounds or ferrocene compounds, etc. can also be used, in addition to potassium ferricyanide.

10 Furthermore, the oxidoreductase is not limited to glucose oxidase shown in the foregoing examples but various enzymes such as alcohol oxidase, cholesterol oxidase, etc. can be used.

INDUSTRIAL APPLICABILITY

15 The biosensor of the present invention can rapidly determine a specific component in various sample solutions in an accurate and simple way. Therefore, its utilization value is extremely high in clinical inspection.

CLAIMS

1. A biosensor for determining a substrate concentration in a sample solution comprising a base plate having an electrode system and a reaction layer having formed thereon a space including said reaction layer, said space being provided with an introducing port for introducing said sample solution into said space and a discharge port for discharging the gas in said space by inflow of said sample solution, said electrode system being equipped with at least an electrode for measurement and a counter electrode, at least an enzyme being carried on said reaction layer, a change in concentration of a substance in the reaction between said enzyme and said sample solution being detected with said electrode system to determine a substrate concentration in said sample solution.
2. A biosensor as claimed in claim 1, wherein said electrode system comprises a plural set of electrode systems and reaction layers corresponding to said plural set of electrode systems and a common space are provided.
3. A biosensor as claimed in claim 1, wherein said electrode system comprises a plural set of electrode systems and, reaction layers and spaces corresponding to said plural set of electrode systems are provided.
4. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for

measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on the surface of said electrode system and said reaction layer comprises an enzyme layer composed of an oxidoreductase and a hydrophilic high molecular substance having formed thereon an electron acceptor layer.

5. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on the surface of said electrode system and said reaction layer comprises an enzyme layer composed of an oxidoreductase and a hydrophilic high molecular substance having formed thereon an electron acceptor layer containing a surface active agent.

6. A biosensor as claimed in claim 4 or 5, wherein said electron acceptor layer comprises fine particles of an electron acceptor having a particle size of not greater than 100 µm.

7. A biosensor as claimed in claim 4 or 5, wherein said hydrophilic high molecular substance is any member selected from hydrophilic high molecular substances of starch, carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride or a mixture thereof.

8. A biosensor as claimed in claim 1, wherein the surface of a material constructing said space is

hydrophilic.

9. A biosensor as claimed in claim 2 or 3, wherein said electrode system comprises two sets of electrode systems comprising at least an electrode for measurement and a counter electrode mainly composed of carbon, a reaction layer composed of a hydrophilic high molecular substance and an oxidoreductase being provided on one electrode system and a hydrophilic high molecular substance layer or a layer composed of a hydrophilic high molecular substance and an inactivated oxidoreductase being provided on another electrode system.

10. A biosensor as claimed in claim 2 or 3, wherein said electrode system comprises two sets of electrode systems comprising at least an electrode for measurement and a counter electrode mainly composed of carbon, a reaction layer composed of a hydrophilic high molecular substance and an oxidoreductase being provided on one electrode system and a hydrophilic high molecular substance layer or a layer composed of a hydrophilic high molecular substance and a deactivated oxidoreductase being provided on another electrode system.

11. A biosensor as claimed in claim 9 or 10, wherein said electrode system comprises an electrode for measurement and a counter electrode mainly composed of carbon and a reference electrode comprising a silver/silver chloride reference electrode.

12. A biosensor as claimed in claim 1, wherein said electrode system is prepared from a material mainly composed of carbon formed on an insulating base plate by means of screen printing.

13. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on said electrode system and said reaction layer comprises a first layer composed of a hydrophilic high molecular substance and an oxidoreductase, a second layer composed of a hydrophilic high molecular substance and a third layer containing an electron acceptor.

14. A biosensor as claimed in claim 13, wherein said hydrophilic high molecular substance in the first layer and the second layer is selected from hydrophilic high molecular substances of starch, carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride or a mixture thereof.

15. A process for preparing a biosensor which comprises forming an electrode system comprising at least an electrode for measurement and a counter electrode on an insulating base plate, coating a hydrophilic high molecular substance aqueous solution and an oxidoreductase aqueous solution on said electrode system and then drying to form an enzyme layer, spreading a mixture of an electron acceptor and an organic solvent onto said enzyme layer,

removing said organic solvent to form an electron acceptor layer and then integrating together with a cover.

16. A process for preparing a biosensor which comprises forming an electrode system comprising at least an electrode for measurement and a counter electrode on an insulating base plate, coating a hydrophilic high molecular substance aqueous solution and an oxidoreductase aqueous solution on said electrode system and then drying to form an enzyme layer, next spreading a solution of a hydrophilic high molecular substance in an organic solvent onto said enzyme layer to form a hydrophilic high molecular substance layer, further spreading a dispersion of an electron acceptor in an organic solvent onto said hydrophilic high molecular substance layer to form an electron acceptor layer, and then integrating together with a cover.

17. A process for preparing a biosensor as claimed in claim 15 or 16, wherein a mixture of said electron acceptor, said surface active agent and said organic solvent is spread on said enzyme layer and said organic solvent is removed to form an electron acceptor layer.

18. A process for preparing a biosensor as claimed in claim 17, wherein said electron acceptor layer comprises fine particles of an electron acceptor having a particle size of not greater than 100 μm .

FIG. 1

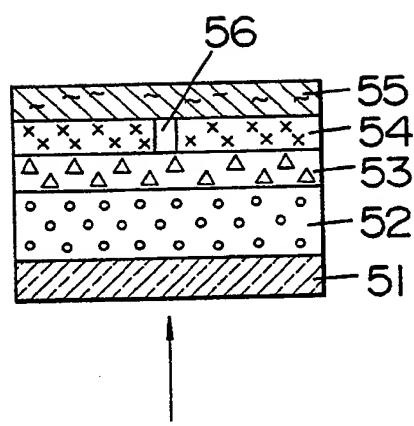


FIG. 2

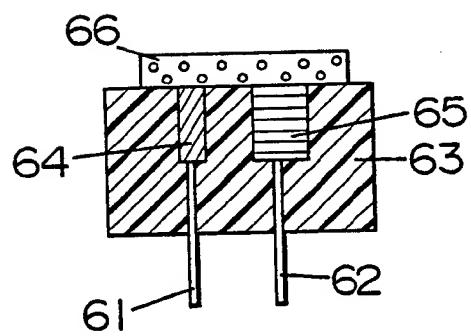


FIG. 3

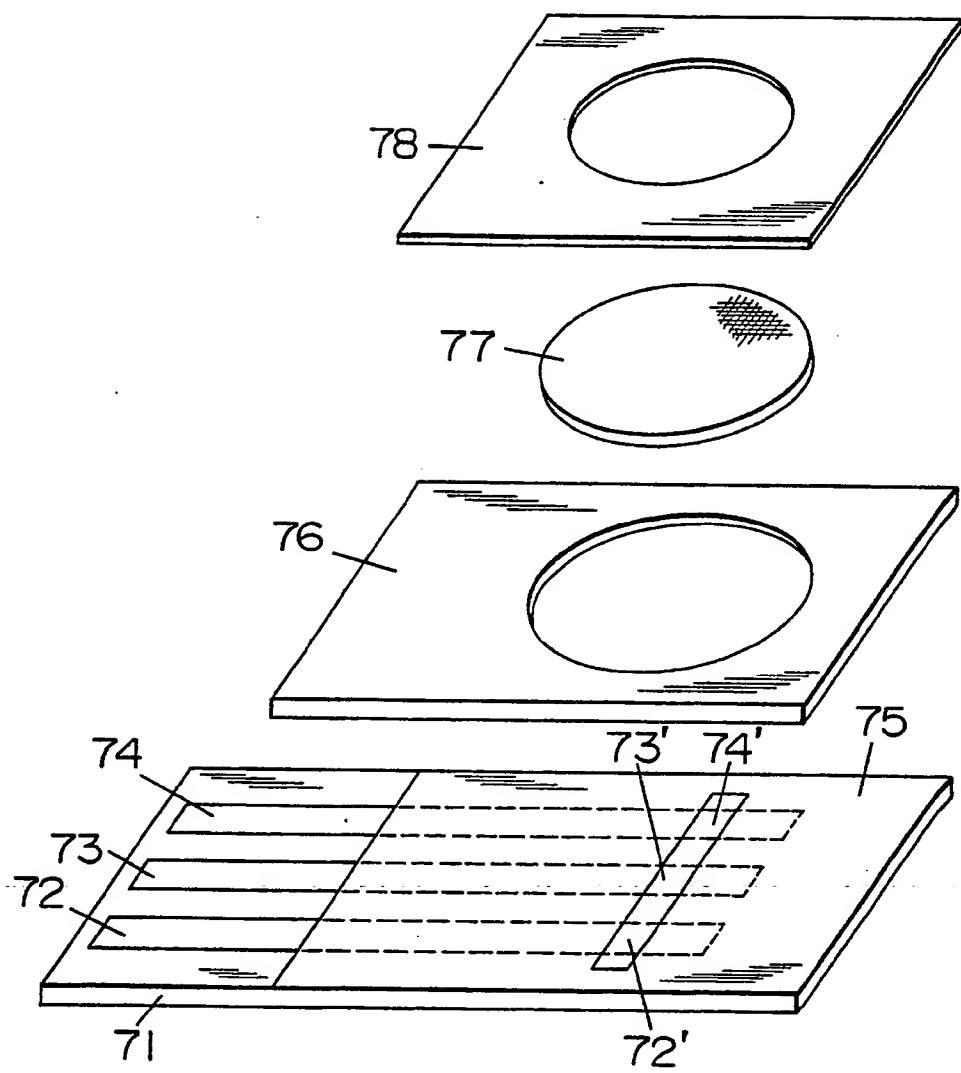
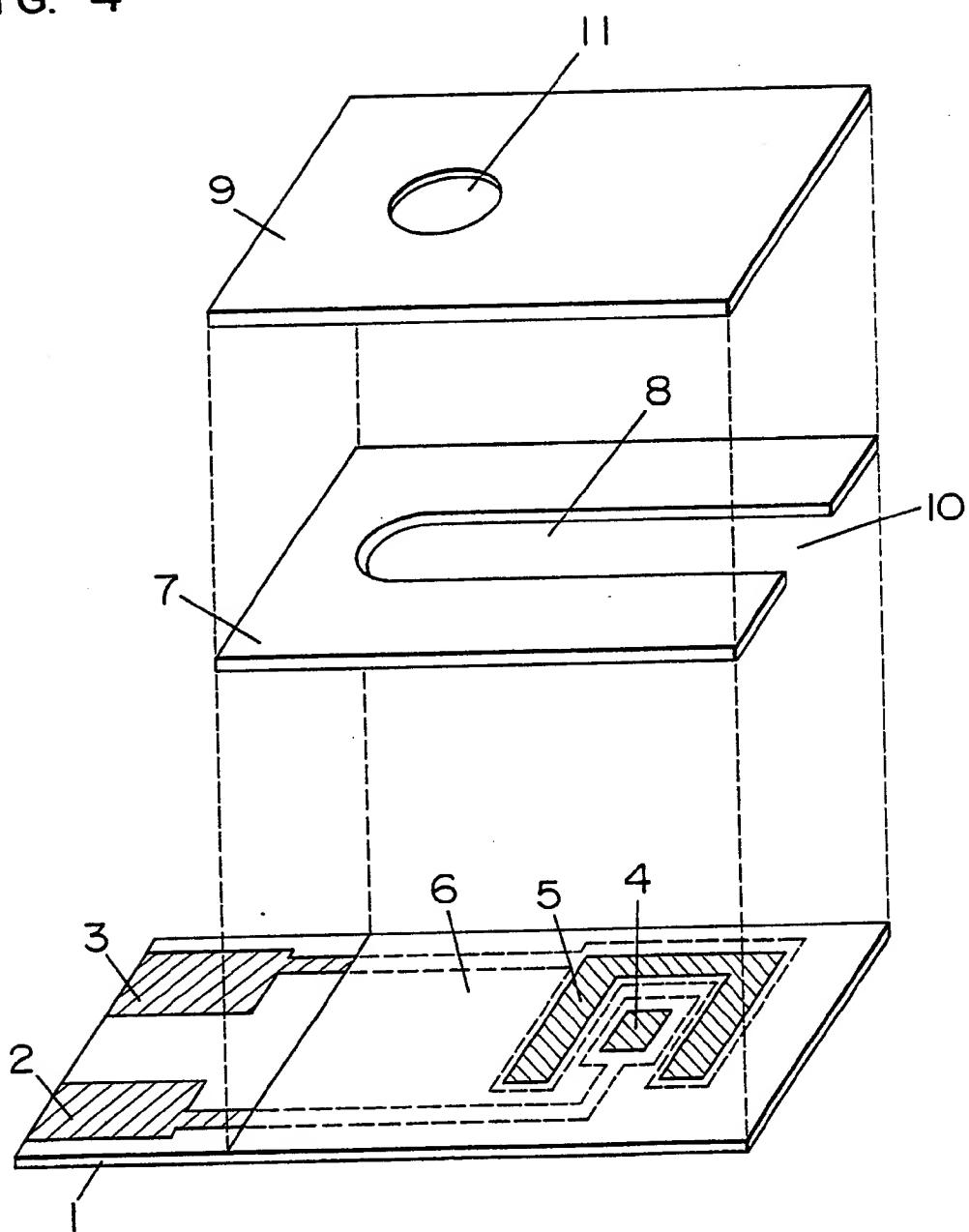


FIG. 4



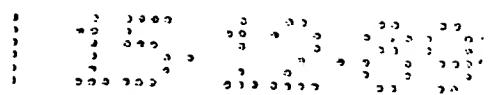


FIG. 5

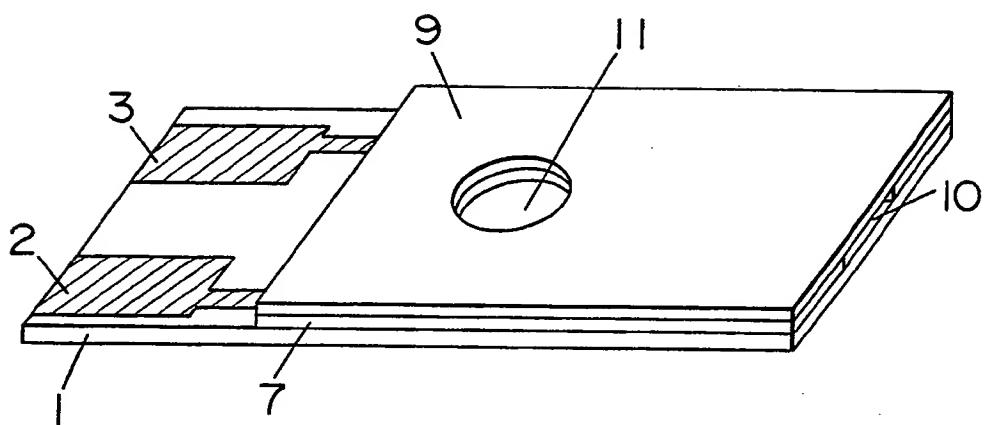


FIG. 6

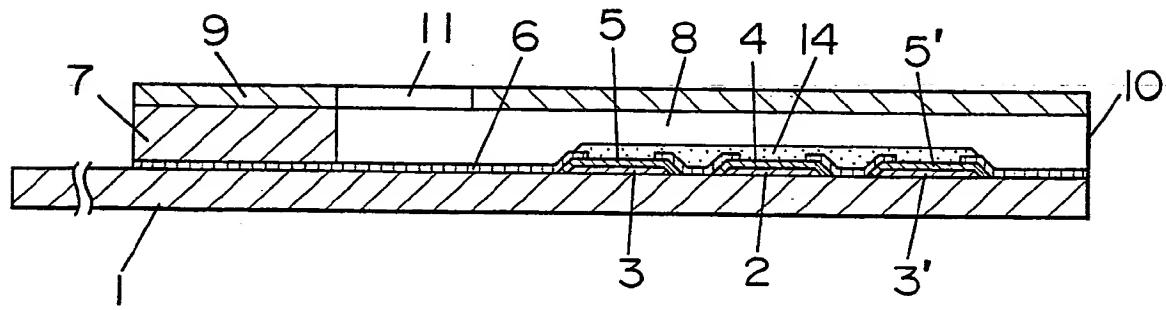
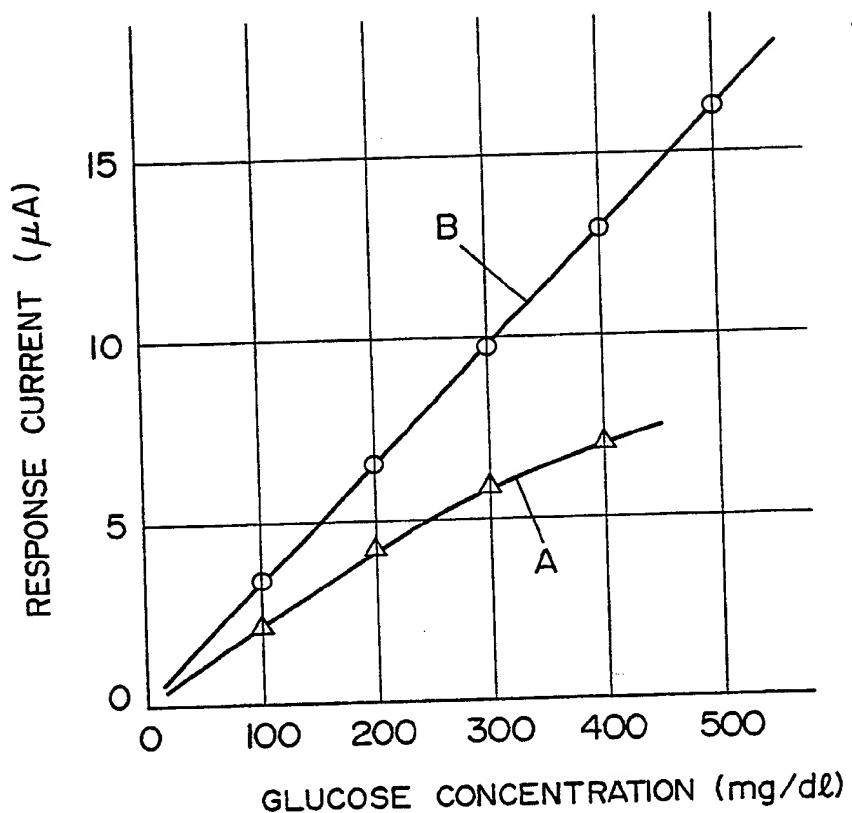


FIG. 7



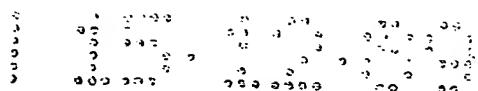


FIG. 8

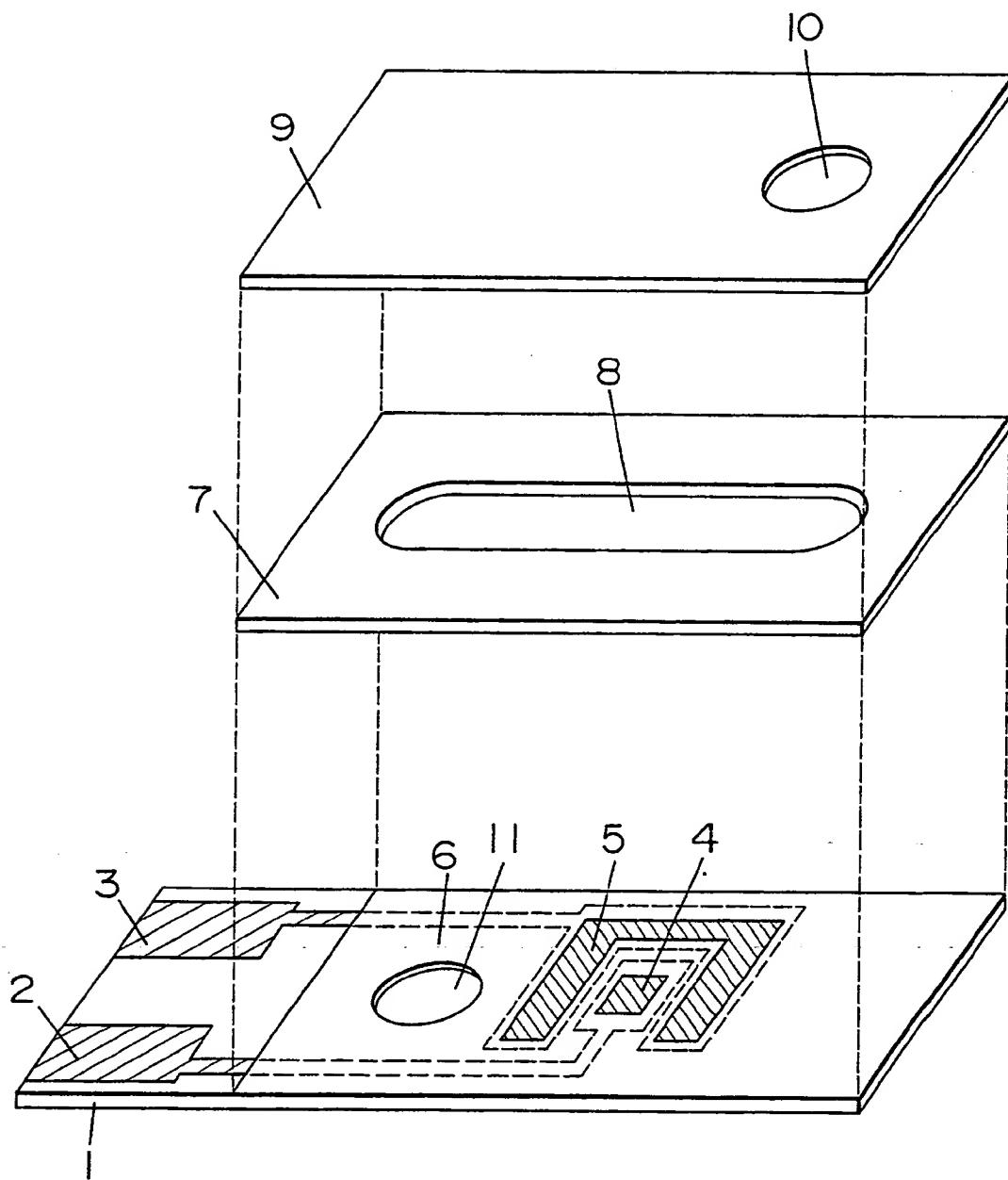


FIG. 9

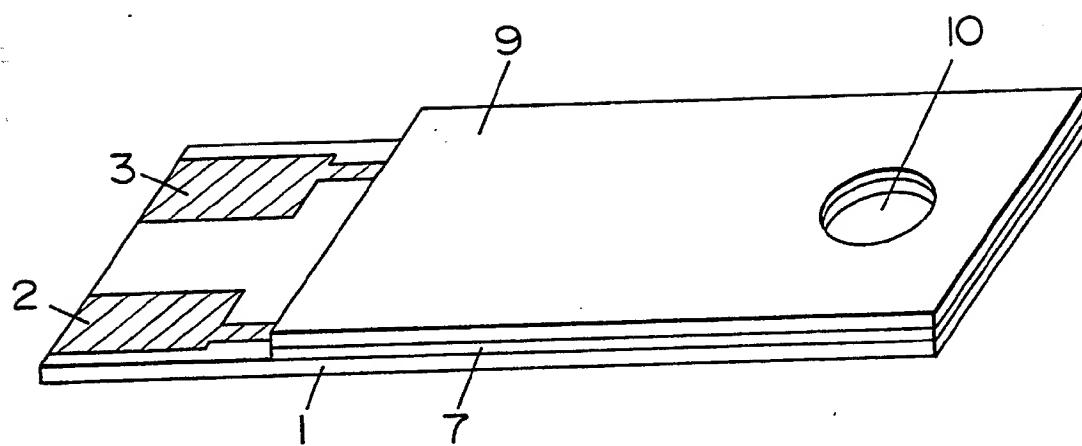


FIG. 10

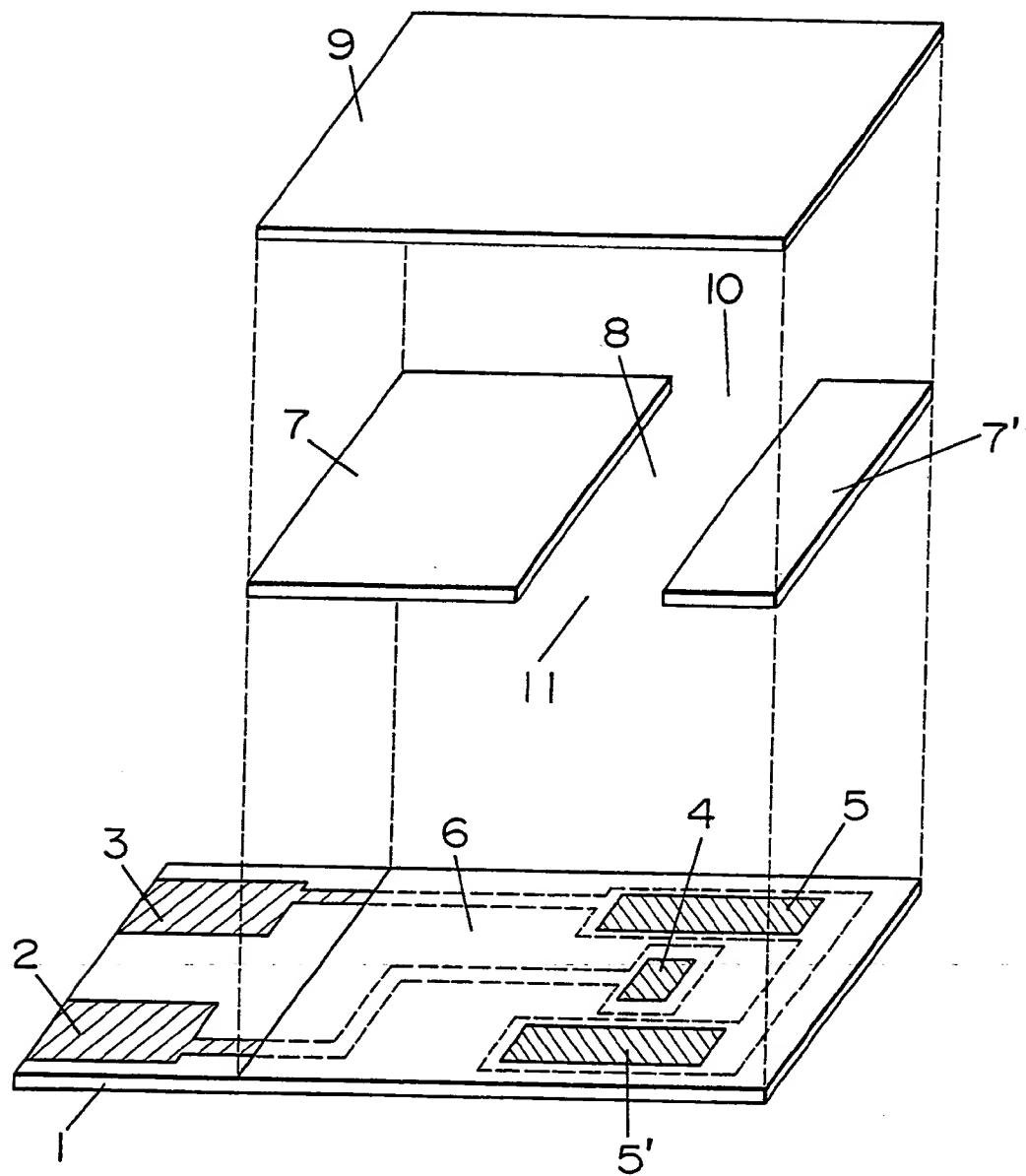
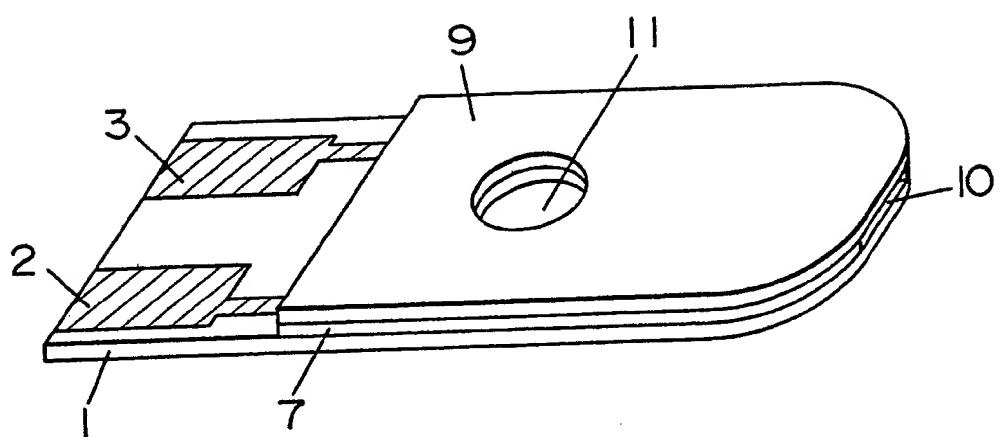


FIG. II



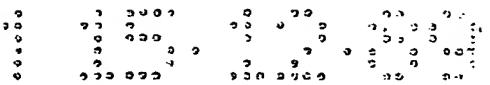


FIG. 12

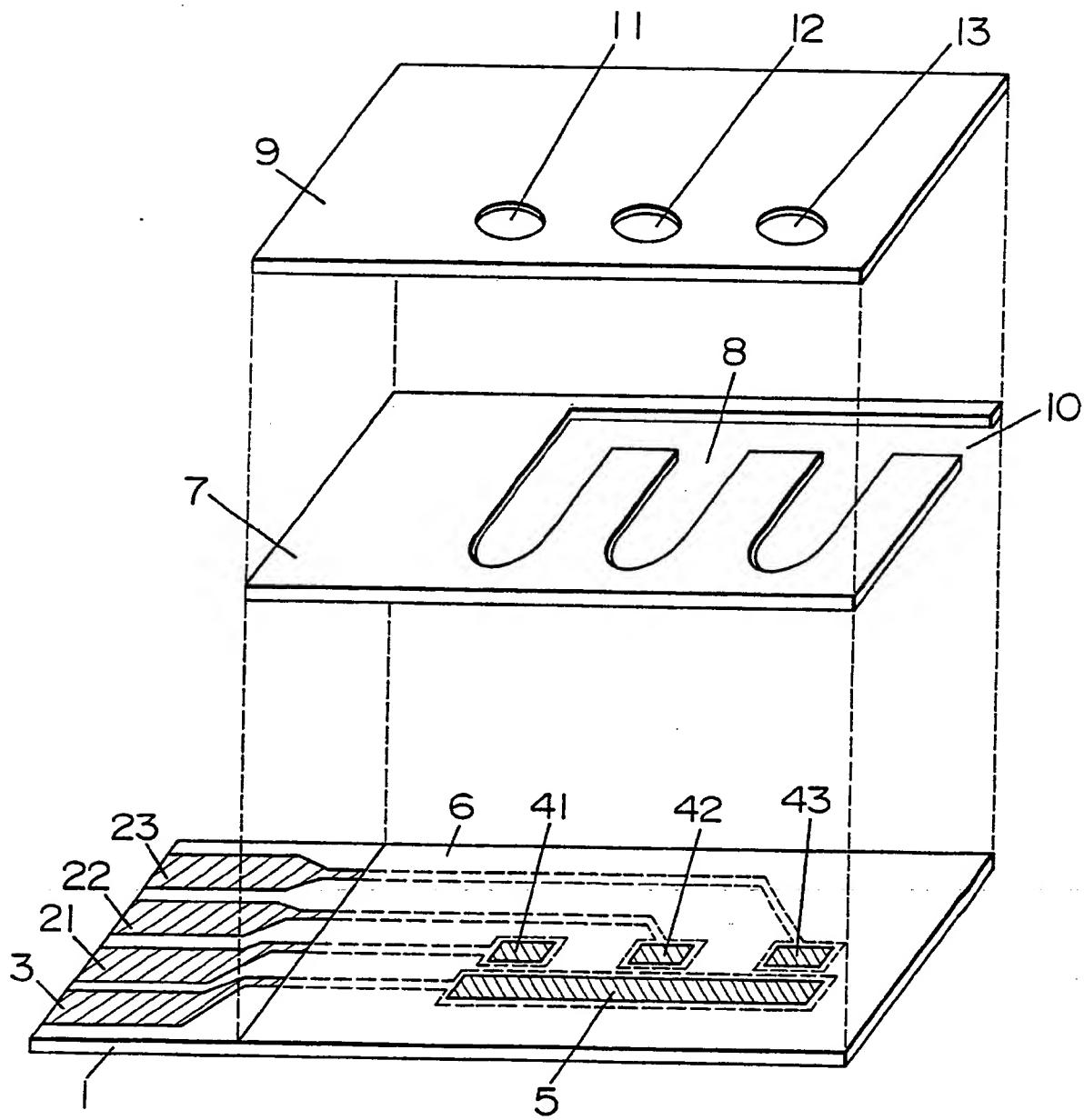


FIG. 13

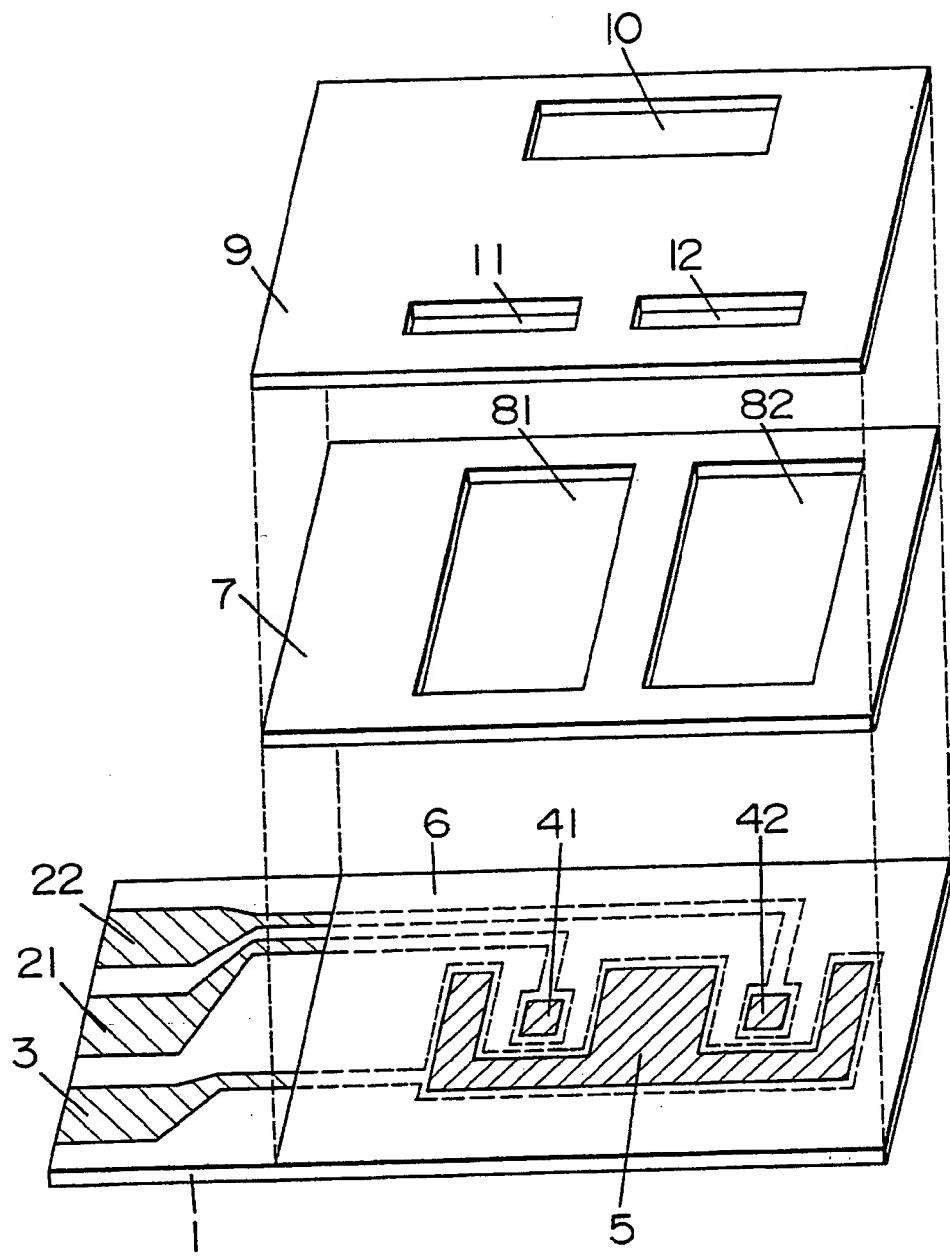




FIG. 14

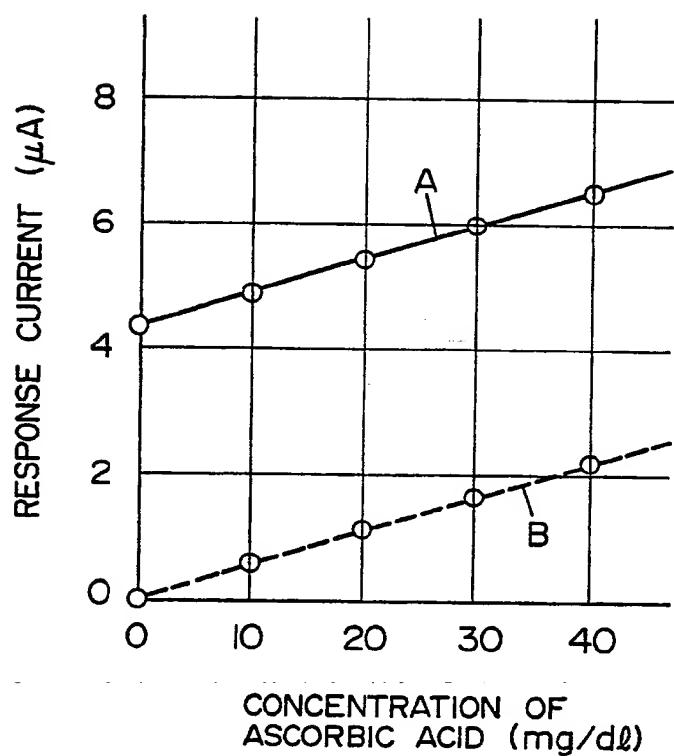
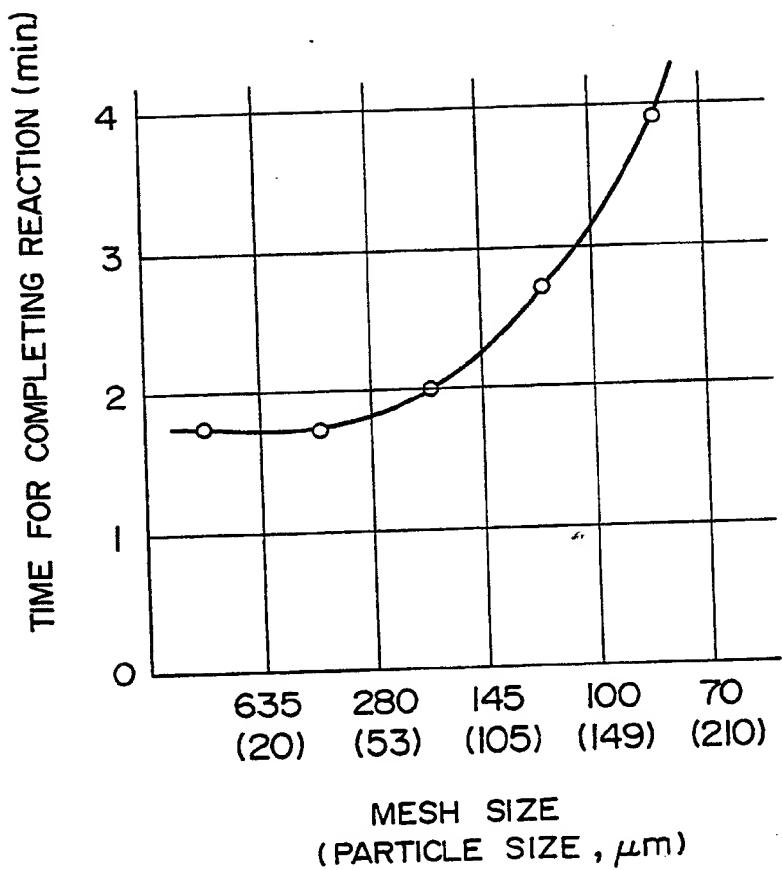


FIG. 15



List of numerals appearing in the drawings:

- 1, 63, 71 base plate
- 2, 3, 3', 21, 22, 23, 61, 62, 72, 73, 74 ... lead
- 4, 41, 42, 43, 64, 73 electrode for measurement
- 5, 5', 65, 72 counter electrode
- 6, 75 insulating layer
- 7, 7' spacer
- 8, 81, 82 space
- 9, 78 cover
- 10 introducing port
- 11, 12, 13 discharge port
- 14 reaction layer
- 51 support
- 52 reagent layer
- 53 spreading layer
- 54 waterproofing layer
- 55 filtering layer
- 55 pore
- 66, 77 porous material
- 74 reference electrode
- 76 holding frame

INTERNATIONAL SEARCH REPORT

International Application No. PCT/JP89/00337

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl⁴ G01N27/28, G01N27/30

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC	G01N27/28-27/30, 27/46

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

Jitusyo Shinan Koho 1926 - 1989
Kokai Jitsuyo Shinan Koho 1971 - 1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	JP, A, 62-137559 (Matsushita Electric Ind. Co., Ltd.) 20 June 1987 (20. 06. 87) (Family: none)	1-18
A	JP, A, 63-3248 (Matsushita Electric Ind. Co., Ltd.) 8 January 1988 (08. 01. 88) (Family: none)	1-18
A	JP, A, 63-58149 (Matsushita Electric Ind. Co., Ltd.) 12 March 1988 (12. 03. 88) (Family: none)	1-18

* Special categories of cited documents: ¹⁰

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
June 26, 1989 (26. 06. 89)	July 10, 1989 (10. 07. 89)
International Searching Authority	Signature of Authorized Officer
Japanese Patent Office	

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